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Mitotic analysis of sticky chromosomes in aluminum tolerant and susceptible wheat lines grown in soils of differing aluminum saturation

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Summary

Aluminum toxicity of acid soils is an important growth limiting factor which can reduce crop yields. Breeders have used various screening techniques for the rapid selection of Al tolerance lines. Al toxicity is complex having multiple effects on plant growth. The question becomes can plant selection for tolerance at one specific Al saturation result in tolerance at multiple aluminum saturation levels. Six near-isolines and three wheat (*Triticum aestivum* L.) cultivars differing in aluminum response on the basis of Al exclusion in the root cell wall were used in this study. Seeds of each line were planted in three soil treatments, 0.5%, 27% and 65% aluminum saturation respectively. Mitotic analysis was carried out on root tip cells. Soils with aluminum saturation were seen to induce sticky chromosome damage in both tolerant and susceptible lines. Lagging chromosomes, chromosome fragments and anaphase bridges were seen in cells of root tips grown in soils with increased aluminum saturation. The number of cells in susceptible lines exhibited an increase in stickiness as the aluminum saturation of the soil increased. In tolerant lines, the number of cells with stickiness increased at 27% saturation, but then decreased at 65% aluminum saturation. The amount of chromosome stickiness appears to be not only dependent upon the plant response to phytotoxic Al but also the aluminum saturation level at which the plant is grown. Tolerance as measured by reduced chromosomal stickiness did not occur until the plants were grown in very high soil Al saturation, indicating a single selection scheme for Al tolerance may not be adequate to develop the highest degree of tolerance.

Introduction

Soil-Al toxicity has been a major concern for crop production throughout the world. Common wheat (*Triticum aestivum*) is no exception. Wheat is the number one food grain consumed directly by humans. More land is devoted to the production of wheat than any other commercial crop in the world (Briggle & Curtis, 1987). A crop of wheat is harvested somewhere in the world every month of the year (Briggle, 1980). The minimum soil pH recommended for wheat production is 5.5. Reductions in grain yield occur when soil pH falls below 5.2 to 5.0. In practice, however, producers often fall short of the target pH of 5.5, either in the subsurface or surface layers of soil or both (Carver & Ownby, 1995). Awareness of soil acidity has increased dramatically in wheat production areas historically regarded as pH safe. Increased demand by wheat farmers for cultivars with improved tolerance to aluminum toxicity has prompted numerous studies to characterize Al tolerance and its genetic control (Carver & Ownby, 1995).

The initial site of aluminum toxicity injury is in the root (McLean & Gilbert, 1927; Clarkson, 1965; Kochian & Shaff, 1991). Once aluminum gains access into the plant, many changes in plant growth can occur. Root growth (Fageria et al., 1988; Foy et al., 1978), respiration (Norton, 1966) and DNA synthesis

(Sampson et al., 1965; Wallace & Anderson, 1982) are all reduced due to Al toxicity. Aluminum can also react with other nutrients in the soil such as P to form less available compounds. In addition, aluminum can interfere with the uptake and transport of substances in plants such as Ca, Mg, P, K and water.

Carver et al. (1993) developed aluminum tolerant near-isolines of the variably sensitive cultivars, Chisholm and Century as part of an effort to transfer aluminum tolerance from soft red winter wheat (Atlas) to hard red winter wheat. In addition, two susceptible near-isogenic lines were also developed. Closely related genotypes (preferably near-isogenic lines) are valuable tools for studying the physiological mechanisms of elemental toxicity or tolerance (Foy et al., 1978). Johnson et al. (1997) indicated that the hematoxylin assay used to identify Al-tolerant wheat isolines may assay external tolerance due to exclusion of Al from root cells and that other types of mechanisms may also exist.

One effect on plants grown in acid soils with phytotoxic aluminum present is the reduction of DNA synthesis. Aluminum not only has been reported to decrease the rate of DNA synthesis, but it also decreases template activity (Morimura & Matsumoto, 1978; Matsumoto & Morimura, 1980). The binding site for aluminum in DNA is phosphorus (Matsumoto et al., 1976). It has been suggested that the binding of aluminum prevents the double helix from separating and serving as the template for RNA synthesis (Matsumoto & Morimura, 1980). The double strands of DNA are captured by the aluminum (Al^{3+}) and are unable to separate. In addition, chromatin fibers can be cross linked by the binding of the Al^{3+} to DNA-phosphate between fibers which results in less active transcription (Matsumoto & Morimura, 1980) and possible interference with cell division.

Levan (1945) and Liu et al. (1995) have both reported that aluminum causes severe cytological abnormalities (such as anaphase bridges) in the dividing cells of onion (*Allium cepa*) roots resulting from chromosomal stickiness. Chromosomal stickiness is defined as a 'chromosomal agglutination of unknown nature which results in a pycnotic or sticky appearance of chromosomes' (Rieger et al., 1976). Caetano-Pereira et al. (1995) also found such a sticky chromosome phenomenon occurring in maize microsporocytes of maize grown in the highly acidic soils of the 'Cerrado' region of the Brazilian plateau. As aluminum saturation in the soil increased, chromosome abnormalities associated with sticky chromosomes also increased. Zannella et al. (1991) demonstrated that the pollen mother cells of wheat plants grown on acid soils with known aluminum saturation showed increased numbers of univalents and micronuclei and increased stickiness of pollen mother cells when compared to the same cultivar grown with pH corrected by lime.

The objective of this study was to determine if aluminum tolerant wheat lines selected by using hematoxylin staining techniques had improved resistance to the sticky chromosomes phenomenon. Mitotic analysis was used to determine the effect aluminum saturation levels had on the dividing cells of young wheat plants, both tolerant and susceptible. If mitotic disruption occurs in Al tolerant lines, the agronomic potential of these lines could be reduced.

Materials and methods

Nine wheat lines were used in this study. Three cultivars, differing in response to aluminum, were Atlas (tolerant to Al), Chisholm and Century (both susceptible to Al). Three near-isolines were derived from Chisholm and selected on their tolerance to aluminum (PI561722 and PI561723) and susceptibility (PI561726) based on visual examination of hematoxylin staining roots (Carver et al., 1993). Three near-isolines were derived from Century and selected on the basis of their sensitivity to aluminum with a hematoxylin stain. Lines PI561724 and PI561725 were selected as tolerant and PI561727 was selected as susceptible. All near-isolines were developed by backcross breeding using Century or Chisholm as the susceptible recurrent parent and 'Atlas 66' as the tolerant donor. The near-isolines differ in their aluminum response, but are noted to have a 97% genetic similarity among themselves and 91% genetic similarity with their recurrent parent (Carver et al., 1993). Seeds were provided by Dr B.F. Carver (Oklahoma State University, USA). Three soils differing in aluminum saturation were used, 0.5%, 27% and 65%. A Porter's soil (coarse-loamy, mixed, mesic Umbric Dystrochrepts) obtained from Tennessee was amended with lime and nutrients to appropriate aluminum saturation levels (Table 1).

For chromosome analysis, three plants of each wheat line grown in the 0.5%, 27% and 65% aluminum saturated soils were used. Kemels were placed in a germination box with 0.5%, 27% and 65% aluminum saturated soil. The germination boxes were placed under continuous light for 24 hrs un-

Table 1. Physical and chemical properties of porter soil (coarse – loamy, mixed, mesic umbric dystrochrepts)[a]

Lime	pH		Exchangeable cations[b]						Al sat. %[c]
g/kg	H_2O	0.01 N $CaCl_2$	Ca	Mg	K	Mn	Al	H	
0	4.3	3.9	0.64	0.09	0.4	0.025	3.18	0.53	65%
2	4.7	4.3	2.03	1.31	0.4	0.030	1.53	0.28	27%
10	5.7	5.4	5.21	4.11	0.4	0.025	0.05	0.32	0.5%

[a] All three treatments received 150, 200, 0.2, 2.0, 10.0, and 10 kg/ha of N, P, Mo, B Cu, and Zn, respectively.
[b] Exchangeable Ca, Mg, K and Mn by 1 M NH_4OAc and Al and H extracted by 1M KCl.
[c] Al saturation % = Al/(Al + H + Ca + Mg + K + Mn) × 100.

der continuous light with an irradiance level of 83 mmole.m^{-2}.sec^{-1} photosynthetically active radiation (PAR) (1400–800 nm) at 21 °C. The boxes were then placed at 4 °C for 24 hr, then returned to continuous light conditions until the roots measured ∼1–2 cm ≈ 2–3 days. The roots were removed from the plants and placed directly into 3:1 ethanol, glacial acetic acid fixative. After five days at 21 °C the roots in the fixative were stored at 4 °C until stained.

Roots were stained as follows: The roots were hydrated for 4 min in distilled water, then hydrolyzed in 5 N HCL for 45 min. After rinsing in ice water, the root tips were placed in Feulgen (leucobasicfuchsin) stain for ∼2 hr, then placed in a Feulgen bleaching solution of 0.05% potassium meta-bisulfite, 0.05 N HCL for 30 min. The roots were then rinsed in distilled water for at least 20 min and then placed in an enzyme solution of 2% cellulysin (Calbiochem) and 1% macerase (Calbiochem) in 0.001 M EDTA. After 45 min, the enzyme solution was removed, and 2–4 drops of 1% acetocarmine placed on the root until slide preparation. The root tips were placed on slides and squashed in 1% acetocarmine.

Two root tips per plant were analyzed. At least two plants were analyzed per line. The number of normal and abnormal anaphase cells per root tip was recorded. A percentage of normal anaphase cells was calculated for each line in each aluminum saturation level.

Results

Mitotic analysis was carried out on all wheat lines grown at the 3 levels of soil Al saturation (Table 2). Anaphase cells were scored as normal or abnormal. An anaphase cell was scored normal when complete separation between the two anaphase nuclei was observed (Figure 1A). An anaphase cell was scored as abnormal if lagging chromosomes, chromosome fragments or anaphase bridges were present (Figure 1B, C, D). Upon statistical analysis, the susceptible and tolerant lines were significantly different with respect to the number of abnormal anaphases and Al saturation ($p > 0.0001$). Both recurrent parents which were reported to be Al susceptible, had the largest number of abnormal anaphases at all Al saturation levels tested. None of the remaining lines had such high numbers of abnormal anaphases at the 27% and 65% Al saturation levels. The non-recurrent parent, Atlas, had overall the most normal anaphase cells. At the 27% Al saturation level, Atlas had the fewest number of abnormal anaphases of any of the remaining wheat lines. At 65% Al saturation only one line, PI561723, was observed to have a higher number of normal anaphases cells than Atlas. With respect to the Chisholm isolines, all of these lines had a higher number of normal anaphase cells as compared to Chisholm at all Al saturation levels tested. The two tolerant lines had a similar number of normal anaphase cells as compared to the susceptible line at 0.5 and 27% saturation but had many more normal anaphase cells at 65% Al saturation. The Century isolines followed a similar pattern. Upon comparison of the tolerant isolines from the two recurrent parents, all had a similar number of normal anaphases at all Al saturation levels. None of the tolerant lines had as many normal anaphases as the nonrecurrent parent Atlas. Two distinct patterns were observed with respect to overall response to Al saturation. All of the susceptible lines had an increase of abnormal anaphases as Al saturation increased while the tolerant lines all had an increase of abnormal anaphase cells at 27% Al saturation and then had a decrease in the number of abnormal cells as the soil aluminum saturation increased to 65% (Figure 2).

Table 2. Mitotic anaphase analysis of wheat lines grown in differing soil aluminum saturation

Line[a]	Aluminum response[b]	% Aluminum saturation	Total anaphases	% Normal anaphases
PI561722^{ch}	T	0.5	229	73%
PI561722	T	27	175	56%
PI561722	T	65	179	72%
PI561723^{ch}	T	0.5	194	70%
PI561723	T	27	140	57%
PI561723	T	65	154	81%
PI561724^{cen}	T	0.5	183	78%
PI561724	T	27	197	57%
PI561724	T	65	234	71%
PI561725^{cen}	T	0.5	174	71%
PI561725	T	27	165	59%
PI561725	T	65	273	69%
PI561726^{ch}	S	0.5	220	80%
PI561726	S	27	216	59%
PI561726	S	65	170	49%
PI561727^{cen}	S	0.5	199	69%
PI561727	S	27	195	54%
PI561727	S	65	197	51%
Chisholm	S	0.5	203	69%
Chisholm	S	27	161	44%
Chisholm	S	65	136	45%
Century	S	0.5	251	68%
Century	S	27	189	42%
Century	S	65	220	36%
Atlas	T	0.5	192	74%
Atlas	T	27	204	65%
Atlas	T	65	226	78%

[a] ch = Chisholm background, cen = century background.
[b] T = Tolerant, S = Susceptible.

Discussion

Abnormal anaphase cells were observed in all of the wheat lines at the 0.5% Al saturation level indicating that there is a background level of sticky chromosomes. The fact that the number of abnormal cells in Chisholm and Century increased with increasing Al saturation is consistent with previous reports on Al phytotoxicity on plants (Levan, 1945; Liu et al., 1995). This Al-dose response of wheat also agrees with the observations of Johnson et al. (1997). Therefore, the two susceptible wheat lines behave in a similar manner with respect to hematoxylin staining, root tip growth, and mitotic chromosome analysis. All three of these techniques agree in their assessment of Al susceptibility. In addition, a similar pattern was also observed in the susceptible isolines.

The susceptible isolines of both recurrent parents had the same dose response. As the soil Al saturation increased so did the number of abnormal cells. However, the number of abnormal cells was not as large as observed in their respective recurrent parents (Table 2). This would indicate that the susceptible isolines had improved Al tolerance despite having the same hematoxylin score as their respective susceptible recurrent parent at 0.72 mM Al as reported by Johnson et al. (1997). Johnson et al. (1997) observed a similar response upon hematoxylin staining at lower

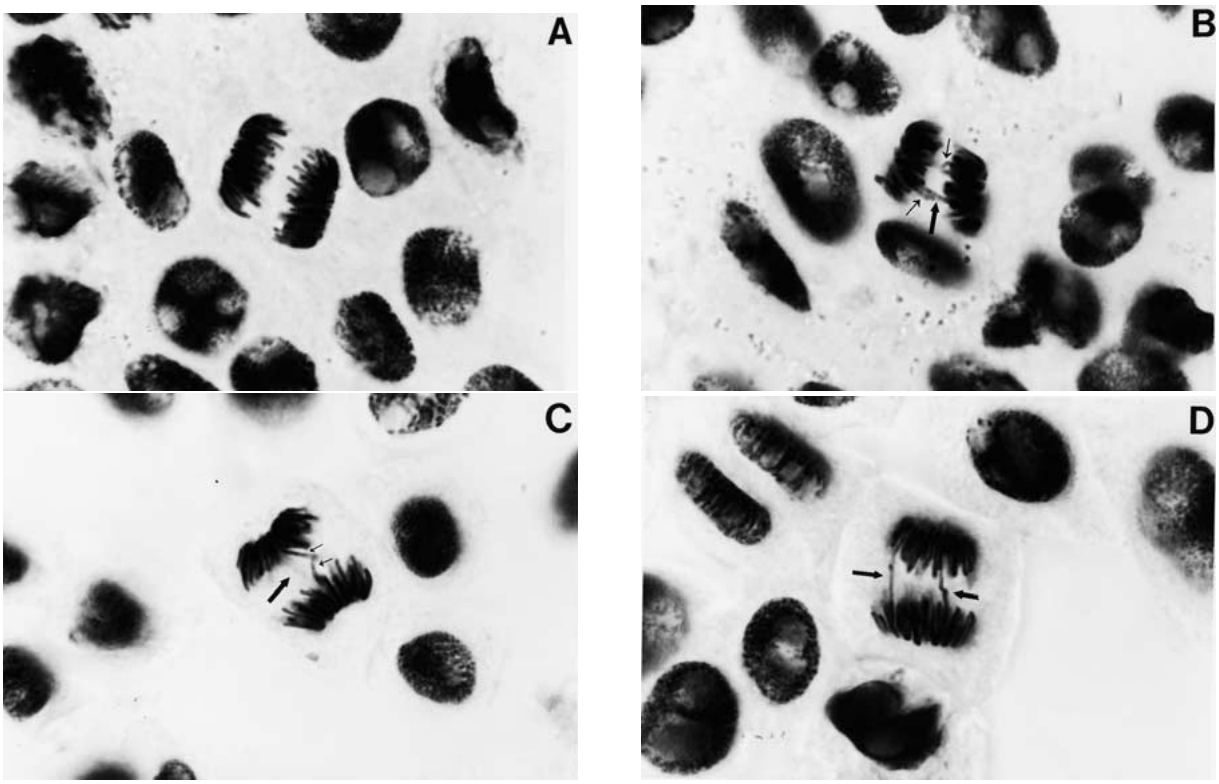

Figure 1. Mitotic Anaphase Cells. (A) A normal anaphase cell with good distribution between the daughter cells. (B, C and D) Examples of abnormal anaphase cells found at higher aluminum saturation levels. (B) Anaphase cell with 1 bridge (large arrow) and lagging chromosomes (small arrows). (C) Anaphase cell with 2 bridges (large arrow) and lagging chromosomes (small arrows). (D) Anaphase cell with 2 bridges (large arrows).

mM Al exposure. Given that the isolines are 91% similar to the parental line, the improvement in the susceptible isolines appears to be due to that portion of the Atlas genome still present in these lines, independent of the hematoxylin staining used to produce these lines. This portion of the genome could contain modifier genes that act on their own to improve Al tolerance or interact with the recurrent parents genome to improve tolerance. Alternatively, an unconscious selection pressure could have occurred during the selection for Al tolerance.

Unintentional selection has occurred in wheat breeding in the past. For instance, the selection for the translocated chromosome 1B/1R in wheat was due in no small part to the increased vigor of those plants carrying this chromosome independent of the specific genes selected (Zeller & Hsam, 1983). By selecting for the best plants from specific crosses, breeders unknowingly selected those plants carrying the translocated chromosome. Such selection in many cases was unrelated to the trait being selected for by the breeder. It is not hard to imagine this type of selection to occur in this case. Unconscious selection for improved germination and growth could result in improved numbers of normal anaphase cells. This is precisely what is seen in both susceptible isolines. By improving the overall background of normal chromosomes, the effect of Al could be somewhat mitigated. This would explain the better than expected performance of the two susceptible lines. Supporting the unconscious selection theory is the observation that both isolines had the same improvement. Given the selection scheme used in developing these isolines, the probability is low that the two isolines would carry the same segments of Atlas by chance or that both isolines would recover the same recurrent background genes resulting in improved cell division.

The tolerant lines all demonstrated a different pattern. In spite of the fact that all of the tolerant lines had a dosage response with respect to mean relative root length (Johnson et al., 1997), this was not reflected in the mitotic analysis. The increase in abnormal cells

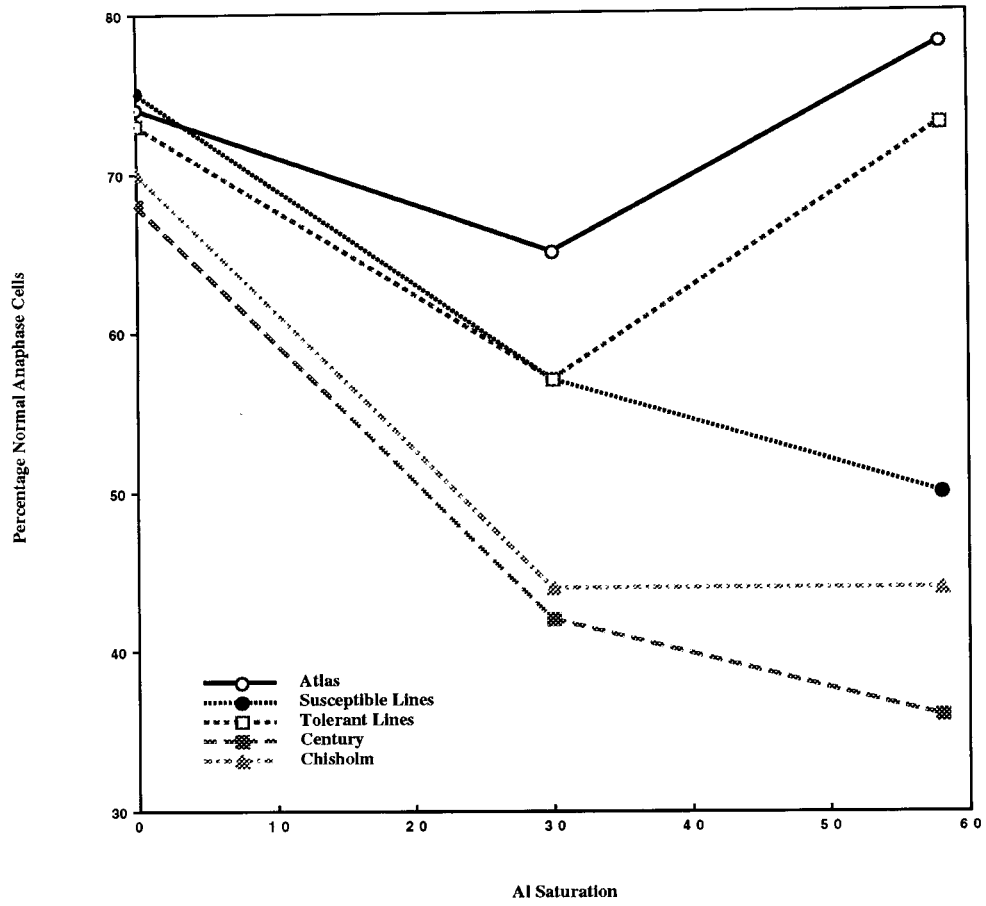

Figure 2. The percentage of normal cells in the wheat lines grown in the three aluminum saturation levels.

at 27% saturation was unexpected. The hematoxylin stain is based on an exclusion of Al from the cells. The results presented here indicate that the exclusion is not completely occurring at the 27% saturation level. Although Atlas, the tolerant donor parent, has fewer abnormal cells, the four tolerant isolines have about the same level of abnormal anaphase cells as the susceptible isolines. With respect to mitotic abnormalities, the tolerant and susceptible isolines are similar in Al response. This is in contrast to the hematoxylin staining data at 0.72 mM but in agreement with staining data at lower molarity as reported by Johnson et al. (1997). The lines classified as susceptible as well as those classified as tolerant all appear to be more Al tolerant than the recurrent parent as discussed previously. This is supported by both the chromosome and hematoxylin stain data. The most probable hypothesis is that genes affecting cell division were inadvertently selected and that improvement of cell division at 27% Al saturation in both the tolerant and susceptible isolines resulted from an overall improvement in cell division over the recurrent parent.

At 65% Al saturation the isolines responded as expected from the hematoxylin staining and mean relative root length assays. Both Atlas and the tolerant isolines had an increase in the normal anaphase cells to at or above the number seen in the 0.5% saturated soil. Thus, the hematoxylin method used to select for Al tolerance appears to be selected for tolerance at very high levels of Al. How the Al saturated soils used in this study compare with acid soils on which crops are grown now becomes an important issue.

The 0.5% Al saturated soil had a pH of $\approx$ 5.7, higher than the minimum recommended pH of 5.5. The pH of the 27% Al saturated soil was 4.7 which is below 5.0, the pH level in which reductions in grain

yield become apparent. The pH of the 65% saturated soil was $\approx$ 4.3, a very low pH which should have major impacts on plant growth. At the highest pH, all of the lines behaved similar indicating that under nonphytotoxic conditions, cell division was occurring in a similar manner. At the intermediate pH, a pH at which one would expect to begin to see Al damage, the tolerant and susceptible isolines behaved similarly and better than their respective recurrent parent, indicating that at this agronomically important level the selection scheme, independent of the hematoxylin screening, resulted in improved cell division and therefore could have enhanced agronomic performance at this pH. The data obtained at the lowest pH level indicate that the hematoxylin staining procedures using 0.72 mm Al does indeed result in selection for Al exclusion and thus an increase in normal anaphase cells to 0.5% saturation levels in the tolerant lines. This is probably due to the inability of the Al to reach the chromatin of the cell.

In conclusion, upon comparing the cytogenetic data with the hematoxylin studies, it is hypothesized that several mechanisms are involved in Al tolerance. One potential mechanism is exclusion thus Al never enters the plant, as demonstrated by the hematoxylin staining, and thus cannot have phytotoxic effects. With no Al reaching the chromatin, no increase in sticky chromosomes would occur. The hematoxylin staining at 0.72 mM effectively selects for this exclusion in highly acidic soils. The second mechanism is improvement of cell division. If an overall increase in the efficiency of mitosis occurred, the amount of overall stickiness would be reduced. The effect of Al on chromatin would be reduced thus providing a secondary means of tolerance. Mitotic analysis could be used as a secondary screen in order to obtain the maximum tolerance. Johnson et al. (1997) reported that complete expression of the Atlas tolerance was not recovered and that other mechanisms may be involved. By combining the two assays, plants with more complete Al tolerance could be obtained.

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